Determination of Ritalinic Acid in Autopsy Material Using SPE and LC/MS/MS

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Abstract

A toddler who had lived with drug addict parents, was found dead. The autopsy could not confirm any unequivocal cause of death. Therefore, a toxicological screening was ordered by the prosecutor. The goal of our examination was to identify or exclude drugs potentially contributing to the death of the child. This screening involved Cloned-Enzyme-Donor-Immunoassay (CEDIA) and Fluorescence-Polarization-Immunoassay (FPIA), a Systematic Toxicological Analysis (STA) using GC/MS and a LC/MS/MS search for amphetamines. All analyses turned out to be blank. In one of the loose tablets seized in the apartment we found methylphenidate and identified it as the branded drug RitalinTM. A hair analysis showed a positive result for methylphenidate. In order to illuminate the ante-mortem application, we re-examined the autopsy material for the major metabolite ritalinicacid, which was not included in the STA. We synthesized ritalinic acid by hydrolysis of methylphenidate in methanolic potassium hydroxide solution. Employing HPLC/DAD the yield was near 100 % (based on their peak areas at 222 nm). The sample preparation is crucial. Due to the amphoteric character of the substance liquid-liquid-extraction fails. The best results were obtained with an optimized Solid-Phase-Extraction (SPE) procedure. LC/MS/MS with electrospray ionization appeared to be the most suitable method for the quantification of both methylphenidate and ritalinic acid. Using the procedure described here neither methylphenidate nor ritalinic acid were detectable in kidney, liver, muscle tissue or in putrescence liquid. Thus, an administration of methylphenidate to the child ante-mortem could not be proven in the autopsy material available.

Introduction

Methylpenidate (α-phenyl-2-piperidineacetic acid methyl ester) is structurally related to amphetamin (β-phenylisopropylamine). It is a milde CNS stimulant and used in the treatment of narcolepsy and attention-deficit/hyper-activity disorder. The main metabolite is the deesterified product, ritalinic acid (α-phenyl-2-piperidineacetic acid).

While methylpenidate is easily analysed by liquid-liquid-extraction and HPLC/ESI-MS/MS, ritalinic acid demands a different sample preparation. We describe the synthesis of ritalinic acid, the optimization of sample preparation and chromatography and the mass spectrometric analysis.

Experimental

0.5 mg Methylphenidate (sigma) was dissolved in 20 µL methanol and 980 µL water in a screw vial (Macherey-Nagel). For hydrolysis, 10 µL KOH (1 M) was added and the vial was incubated at 80 °C.

The reaction product was analysed with a Shimadzu HPLC system (2 LC-10AD pumps, SPD-M10A DAD, SCL-10A controller) with an Agilent Zorbax XDB-C8 (4.6 x 150 mm; 5 µm particle size) column. Solvent was acetonitrile/sodium phosphatebuffer pH 2,3 (3/5, v/v).

For HPLC/MS/MS an Agilent 1100 series system with a Macherey-Nagel CC 125/2 Nucleosil 50-5 C 18 ec column and a Waters quattro micro™ triple quadrupole mass spectrometer with an electrospray ion source were employed. Solvent A was water with 0.1 % formic acid, and solvent B was acetonitrile with 0.1 % formic acid.

2 g human kidney tissue was mixed with 8 mL sodium phosphate buffer (pH 6) and homogenized. This homogenate was spiked with ritalinic acid, N-phenyl anthranilic acid and tryptophan at 0.01 mg/mL, 1.0 mg/mL and 0.01 mg/mL, respectively. The spiked homogenatewascentrifuged10 min at 3000 rpm, andthe supernatant was subsequently centrifuged 4 min at 10 800 rpm. This supernatant was used for SPE.

SPE columns tested were Clean-Up® CEC18123/200 mg, Clean Screen® CSDAU203/200 mg, Clean Screen® CSDAU131/130 mg (UnitedChemical Technologies, Inc.); abselut®NEXUS/60 mg, SPEC®DAU/30 mg (Varian); Oasis®MAX/30 mg, Oasis®MCX/30mg (Waters).The anion exchange column MAX was tested in combination with the cation exchange column MCX. MCX was tested alone, too.

All columns were washed with methanol before loading. After loading, MAX was washed with 5 %Ammonia and methanol, MCX with 2 %formic acid and methanol, and all others with 0.1 M acetic acid. MAX was eluted with 2 % formic acid in methanol, MCX with 5 % conc. Ammonia in methanol, and all others with dichlormethane / isopropanol / conc. ammonia (80/20/2, v/v/v).

Extracts were dried 60 °C under N_2-flow. The residues were reconstituted with acetonitril/water (1/1, v/v) for HPLC/MS/MS.

Results and Discussion

Hydrolysis of methylphenidate was monitored over a period of 2 h in time steps of ½ h. Reaction times longer than ½h did not result in larger yields, but in an increase of byproducts (Fig. 1 and 2).

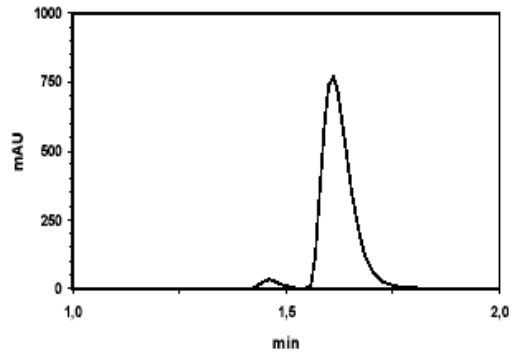

Fig. 1: Chromatogram of hydrolysis mixture (t = 0): methylphenidat and ritalinic acid

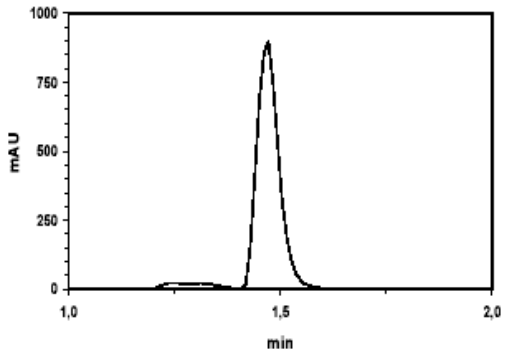

Fig. 2: Chromatogram of hydrolysis mixture (t = 0.5 h): ritalinic acid and other reaction products

An instrument controlled gradient was developed that started with 80 % A at 0 min, 2 min hold, to 10 % A at 5 min, 3 min hold, to 80 % A at 10min, 2 min hold. RT: 1.23 min (tryptophan), 1.65 min (ritalinic acid) and 7.96 (N-phenyl anthranilic acid).

Selected fragmentations for Multiple Reaction Monitoring (MRM): methylphenidate: m/z 234.0 > 55.9 (Target) and > 83.7 (Qualifier); ritalinic acid: m/z 220.0 > 83.7 (T) and > 173.9 (Q); N-phenyl anthranilic acid: m/z 213.9 > 166.9 (T) and > 195.9 (Q); tryptophan: m/z 204.9 > 145.9 (T) and > 187.9 (Q).

Best results for SPE from spiked kidney tissue were obtained with Oasis®MCX and SPEC®DAU. In a closer inspection four extractions were made with each column type. As a result, the mean peak areas were larger in MCXsamples than in DAU samples: 143 x 103vs. 60 x 103 (ritalinic acid) and 55 x 103vs. 9 x 103(N-phenyl anthranilic acid). Coefficients of variation were much smaller in MCX samples than in DAU samples: 1.4 vs. 49 (ritalinic acid) and 0.2 vs. 36 (N-phenyl anthranilic acid).

N-phenyl anthranilic acid can be used as an internal standard. Relative high concentrations are needed, though. Peak are as of tryptophan were up to one order of magnitude higher than peak areas of ritalinic acid, even when there was no spiking with tryptophan at all. The kidney tissue appears to be a strong tryptophan source, so tryptophan disqualifies for an internal standard substance. Having in mind possible ion suppression effects in the ESI source, it is useful to include tryptophan in the MS/MS method to make sure there is no coelution of ritalinic acid and tryptophan.

In the autopsy material available neither methylphenidate nor ritalinic acid were detectable. Thus, an administration of methylphenidate to the child ante-mortem could not be proven.

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